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Effects of Gonadotropin Releasing Hormone (GnRH) on bovine spermatogonial stem cell proliferation

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Abstract

Identification of exogenous factors affecting spermatogonial stem cells (SSCs) proliferation *in vitro*, provides worthy ways to study the basic biology of the cells. The aim of this study was to investigate the effects of a GnRH analogue (alareline acetate) on SSCs colonization in short-term co-culture with sertoli cells. Five, three-month old Holstein male calves were used to isolate spermatogonial and sertoli cells. Testicular germ cell collection was made by enzymatic digestion methods. The cells were co-cultured in a 15 day period and *in vitro* effects of various doses (0.5, 1, 2 and 4 µg/ml) of GnRHa on SSCs colonization were assessed. Effects of GnRHa on SSCs proliferation were dose dependent. In conclusion, it was demonstrated that 1 µg/ml GnRHa was the optimum dose for SSCs colonization in comparison with control group. The highest treatment dose (4 µg/ml GnRHa), negatively affected SSCs colonization in comparison with control group.

Keywords: GnRH, Calf, Spermatogonial stem cell, Sertoli cell, Co-culture

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Introduction

Spermatogenesis is an organized process consisting of complex sequential steps of cell proliferation and differentiation on the basis of SSCs. SSCs are the only cells in postnatal mammals that self-renew and transfer genes to the next generation (Kubota and Brinster, 2006). Stem cells are the ones that can self-renew as well as producing one or more mature type cells during differentiating process. A stem cell can make two stem cells (self-renewing division), one stem and one differentiated cell (asymmetric devision) or two differentiated cells (differentiating devision). Yet, there is little knowledge how each devision is regulated (Izadyar *et al*., 2003).

SSCs are particularly important in cattle, which have a long generation interval compared to laboratory animals and smaller species of livestock. A system in which genes could be delivered to cattle through SSCs would considerably shorten the time to obtain transgenic animals (Khaira *et al*., 2005; Ryu *et al*., 2005; Hermann *et al*., 2007).

According to Izadyar *et al*. (2002), great population of sertoli and spermatogonial cells can be achieved from 3-5-month-old calves. At this age, the testes have a great pool (65-87%) of type A spermatogonia cells.

In general, SSCs colonization *in vitro* is useful for *in vitro* enrichment and purification of these cells for further studies on cryopreservation, restoration of male fertility by transplantation of SSCs in cancer survivors who have undergone chemotherapy and radiation treatments, genetic modification, gene transfection, contraceptive strategies, rare animal protection, *in vitro* differentiation, etc (Meachem *et al*., 2001; Ryu *et al*., 2005; de Rooij., 2006; Hermann *et al*., 2007).

For several reasons, sertoli cells play vital roles in the spermatogenic function of the testis. These somatic cells generate and maintain the cytoarchitecture of the germinal epithelium and blood-testis barrier. Sertoli cells synthesis and secrete some substances such as pyruvate, androgen binding protein - (ABP), estrogen, tubular fluid, 5-dihydro testosterone (DHT), stem cell factor (SCF), glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF) and GnRH like peptide (Griswold, 1995; Dym and Cavicchia, 1997).

GnRH is a hypothalamic neuronal secretory decapeptide that plays an important role in mammal's reproduction. Main source and target site for GnRH are hypothalamus and pituitary gland respectively, but several reports have recently suggested extra-hypothalamic GnRH and GnRH-receptors (GnRH-Rs) in various tissues such as ovaries, placenta, endometrium, oviducts, testes, prostate, mammary glands, spinal ganglia, pancreatic islet, granulose, adrenal gland and immune system cells in rats, rabbits, pigs, cows, monkeys and humans (Bourne *et al*., 1980; Koko *et al*., 1995; Bull *et al*., 2000; Millar, 2005; Ramakrishnappa *et al*., 2005). Hypothalamic GnRH can not be the ligand of the testis GnRH-Rs which are located on leydig and germ cells, because GnRH concentration in the general circulation is very low and it's half-life is short. Because of the adjacent anatomical relationship between sertoli and germ cells, secreted GnRH by sertoli cells seems to have a paracrine role in spermatogenesis, maturation or sperm fertilization ability, although it's strict role(s) on bovine SSCs is not understood.

The aim of the present study was assessment of the effects of various doses (0.5, 1, 2 and 4 μg/ml) of a GnRHa (alareline acetate) on bovine SSCs colonization in short-term co-culture with sertoli cells.

Materials and methods

Animals

Five three-month old Holstein male calves from Aminabad Research Institue, University

of Tehran (Tehran, Iran), were used.

Sertoli and spermatogonial cells collection

The research was conducted in accordance with Local Bioethics Committee of Veterinary Faculty of Tehran University. Testicular biopsies were done after calves sedation with xylazine hydrochloride (Rompun, Bayer AG, Leverkusen, Germany), local anesthesia and aseptic preparation. Testicular Sperm Extraction (TESE) method was used to sample collections. Samples were placed in Dolbecco's Modified Eagle Medium (DMEM; Sigma Chemical Co, St Louis, MO, USA) and transferred to laboratory, beside ice within an hour. Testicular germ cells collection was done with enzymatic digestion according to Izadyar *et al*. (2003) with little modification. Briefly, obtained testis pieces were mechanically minced and floated in DMEM containing 1 mg/ml collagenase, 1 mg/ml trypsin, 1 mg/ml hyaluronidase type II and 5 µg/ml DNase I (all from Sigma Chemical Co, St Louis, MO, USA) and then incubated at 32$^{\circ C}$ for 60 min. After three times being washed for in DMEM and 10% Fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and excluding the interstitial cells, for secondary digestion step, seminiferous tubules were incubated in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase type II and 5 µg/ml DNaseI for 15 min. Finally, to achieve favorite cell population after three washings, the obtained cellular suspension was centrifuged at 30 g for 2 min.

Spermatogonial cells co-culture with sertoli cells

Because of the important role of sertoli cells in SSCs proliferation, co-culture method was used in the present study. Samples were obtained from prepubertal calve's testis (3-5 month-old). At this age, only sertoli and primitive spermatogonial cells are present in the seminiferous tubules. Therefore, after the second enzymatic digestion step, only sertoli and primitive spermatogonial cells are present in the specimen. Following the second enzymatic digestion, 25cm^2 flasks that were coated with

5 µg/ml Datura stramonium agglutinin-lectin (DSA; Sigma Chemical Co, St Louis, MO, USA) in phosphate-buffered saline (PBS) were used for sertoli cells isolation. Cell suspension, obtained from secondary enzymatic digestion, was placed on the coated flasks. The flasks then were incubated at 37$^{\circ C}$ for an hour in a humidified atmosphere containing 5% CO_2. After passing the incubation period, non adherent cells were collected. Sertoli cells formed a feeder monolayer cells after three days incubation in a media containing DMEM plus 10% FBS. In the next step of the experiment, spermatogonial cells were co-cultured in five groups, for 15 days. SSCs co-culture was done in a medium containing DMEM plus 10% FBS, 100 ng/ml GDNF, 100 IU/ml penicillin and 100 mg/ml streptomycin. Experimental groups were: Control (without GnRHa), A (0.5 µg/ml GnRHa), B (1 µg/ml GnRHa), C (2 µg/ml GnRHa) and D (4 µg/ml GnRHa). Supplemented media cultures with mentioned doses of GnRHa were refreshed every 3 days. Five replications were done for each experimental group.

Sertoli and spermatogonial cells identification

Immunocytochemistry staining and cell morphological analysis methods were used to identify sertoli and spermatogonial cells. For the immunocytochemistry staining, vimentin and Oct-4 were detected in sertoli and SSCs, respectively. Vimentin was detected in sertoli cells according to procedure described by Anway *et al*. (2003) and Tajik *et al*. (2010). After an overnight culture, cells were fixed and permeabilized in acetone at -20$^{\circ C}$ for 2 min and then kept at 4$^{\circ C}$ for 30 min. Slides were then rinsed three times with Tris-buffered saline (TBS; Sigma Chemical Co, St Louis, MO, USA) containing 5% Bovine serum

albumin (BSA; Sigma Chemical Co, St Louis, MO, USA). Then, diluted anti-vimentin (Abcam, Cambridge, UK) in TBS/BSA (5 µg/ml and 2 µg/ml respectively) was applied over slide at room temperarure for 60 min. The slide was washed with TBS/BSA and then, diluted Fluorescein isothiocyanate conjugated with sheep anti-mouse IgG (Sigma Chemical Co, St Louis, MO, USA) in TBS/BSA in a ratio of 1:50 was applied on the slide; incubation continued at the room temperature for 45 min. Following slide rinsing with TBS/BSA, the nuclei were counterstained by 4, 6 diaminido-2-phenylindole dihydrochloride (DAPI; Calbiochem, Nottingham, UK) at 0.1 µg/ml for 5 min. The slide was then washed by TBS/BSA and mounted in PBS/glycerol 90% and was observed under a fluorescence microscope (Olympus, Tokyo, Japan). More than 90% of the feeder layer cells were stained by anti vimentin. Cell morphological analysis confirmed this purity of the sertoli cells too.

Kubota *et al*. (2004) demonstrated Oct-4 as a marker for undifferentiated cells. SSCs colonies were immunocytochemically stained with anti Oct-4. The cells were fixed and permeabilized in acetone at $-20^{\circ C}$ for 2 min and then were kept at $4^{\circ C}$ for 30 min. The slide was then rinsed three times with TBS containing 5% BSA. Diluted anti Oct-4 (Abcam, Cambridge,UK) in TBS/BSA was applied over the slide at room temperature for 60 min. Slide rinsing was performed with TBS/BSA and then FITC-conjugated with donkey polyclonal secondary antibody to goat IgG (Sigma Chemical Co, St Louis, MO, USA) was added and incubation was performed at room temperature for 45 min. Following slide rinsing with TBS/BSA, the nuclei were counterstained by DAPI at 0.1 µg/ml for 5 min. After being washed with TBS/BSA, the slide was mounted in PBS/glycerol 90% and was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Morphologically, sertoli and SSCs are pyramid like and spherical shape, respectively. Bovine SSCs colonies on the feeder monolayer sertoli cells were round in shape, with distinct margin and brown in colour which is in agreement with Narenji Sani *et al*. (2012a).

Colony assay

The number and surface area of the spermatogonial cell derived colonies were measured on days 3rd, 6th, 9th, 12th and 15th after culture according to Narenji Sani *et al*. (2012a). For the measurements, inverted microscope (Olympus, Tokyo, Japan) equipped with ocular grid was used.

Statistical analysis

The data were analysed using SPSS statistical software, 2010, Version 16.0. IBM Corporation USA. The results are expressed as mean±SE. The statistical significance between mean values was determined by One Way Analysis of Variance (ANOVA) and Duncan post hoc test; $p < 0.05$ was considered significant.

Results

The results of immunocytochemistry staining for vimentin and Oct-4 are shown in Fig.1. Vimentin is expressed in sertoli cells, while Oct-4 is expressed only in SSCs.

The number and surface area of the SSCs colonies were assessed on days 3rd, 6th, 9th, 12th and 15th after culture. The numbers of the SSCs colonies (Mean±SE) for different concentrations of GnRHa in the media culture are shown in Table1.

Surface areas data of the SSCs colonies (Mean±SE) for different concentrations of GnRHa in the media culture are shown in Table 2.

Groups, based on GnRHa concentration in the media culture: control (0 µg/ml GnRHa), A (0.5 µg/ml GnRHa), B (1 µg/ml GnRHa), C (2 µg/ml GnRHa) and D (4 µg/ml GnRHa).

The average results from five replications were used for control and each experimental group.

Values in the same column with different

superscripts are significantly different (p< 0.05).

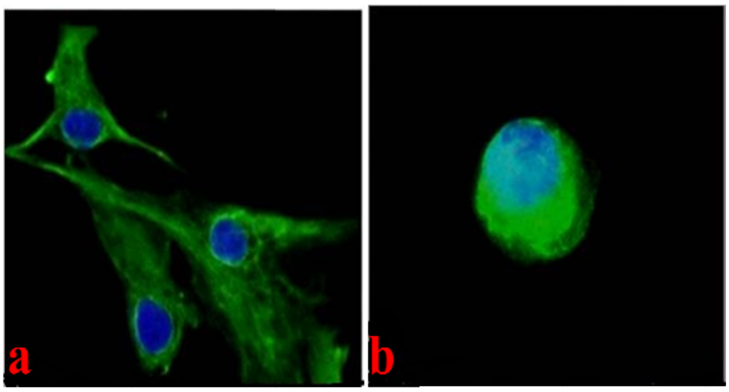


Figure 1. Immunocytochemical staining of sertoli and SSCs. (a) Vimentin was detected in the feeder monolayer cells (sertoli cells). (b) Oct-4 positive colony. Nuclei were counterstained with DAPI. Magnification: ×400

Table 1. Comparison of colony counts between control and experimental groups at different times. Data are presented as mean±SD

	Days after culture				
Groups	**3**	**6**	**9**	**12**	**15**
control	13.5±1.5^{a}	12.25±1.8^{ab}	5.5±1.1^{a}	8.75±1.6^{a}	9.5±2.2^{a}
A	15.2±2.2^{a}	11±2.5^{ab}	12.2±2.1^{a}	11.4±1.5^{a}	16±2.4^{a}
B	88.2±20.6^{b}	54.6±13.3^{c}	94.2±23.5^{b}	71.6±20.9^{b}	89.8±25.3^{b}
C	45.6±13.6^{a}	38.8±14.7^{bc}	83±33^{b}	60.2±23.3^{b}	37.2±13^{a}
D	12.4±2.7^{a}	6±0.8^{a}	7.6±1.4^{a}	4±0.4^{a}	6.8±0.8^{a}

Groups, based on GnRHa concentration in the media culture: control (0 µg/ml GnRHa), A (0.5 µg/ml GnRHa), B (1 µg/ml GnRHa), C (2 µg/ml GnRHa) and D (4 µg/ml GnRHa).

Table 2. Comparison of colony surface area (mm^2) between control and experimental groups at different times. Data are presented as mean±SD

	Days after culture				
Groups	**3**	**6**	**9**	**12**	**15**
control	108.95±3.9^{ab}	59.15±12.2^{a}	79.19±23.4^{b}	35.29±2.2^{a}	48.02±6.3^{a}
A	129.07±12.2^{b}	57.19±9.9^{a}	81.46±16.9^{b}	76.19±13.5^{ab}	41.85±3.3^{a}
B	98.8±12.2^{ab}	110.29±6.8^{b}	88.42±7.1^{b}	125.46±20.2^{c}	145.68±22.9^{c}
C	91.18±9.3^{a}	75.9±6.5^{a}	66.84±4.7^{ab}	86.30±16.6^{bc}	98.30±17.6^{b}
D	91.01±6.3^{a}	78.7±15.1^{a}	32.16±4.7^{a}	46.52±9.1^{ab}	30.95±1.2^{a}

The number of the SSCs colonies in group B (1 µg/ml GnRHa) was significantly higher than control group on all days (p< 0.05). When 2 µg/ml GnRHa (group C) was added to the media culture, the number of the SSCs colonies was higher than control group on days 9th and 12th (p< 0.05), although the number of the SSCs colonies in group B was higher than group C on all days. In comparison with control group, supplementation of media culture with 0.5 µg/ml GnRHa (group A) could not increase the number of the SSCs colonies. Media culture supplementation with 4 µg/ml GnRHa (groupD) decreased the number of the SSCs colonies in comparison with control group, although the difference was not significant.

In the second step of the experiment, surface area of the SSCs colonies was measured on 3rd, 6th, 9th, 12th and 15th days after culture (Table 2).

When 1 µg/ml GnRHa (group B) was added

to the media culture, in comparison with control group, surface area of the SSCs colonies was increased ($p<0.05$). Treatment increased surface area of the SSCs colonies when 2 µg/ml GnRHa was added to the media culture (group C), in comparison with control group on days 12th and 15th ($p<0.05$). In comparison with control group, supplementation of media culture with 0.5 µg/ml GnRHa (groupA) did not have great effect on surface area of the SSCs colonies. It seems that media culture supplementation with 4 µg/ml GnRHa (group D) inhibited SSCs colonization in comparison with control group.

Discussion

In the present study it was demonstrated for the first time, that GnRHa affects *in vitro* proliferation of bovine SSCs in a dose dependent manner. SSCs proliferation and differentiation in prepubertal testis depend on production of several secreted factors by sertoli cells located in the seminiferous tubules adjacent to the cells (Pellegrini *et al*., 2003). One of the factors that is secreted by the sertoli cells is a GnRH like peptide but to the date, effect(s) of the peptide on SSCs is unknown (Griswold, 1995). In the present study effects of various doses (0.5, 1, 2 and 4 µg/ml) of a GnRHa (alareline acetate) on bovine SSCs colonization in short-term co-culture with sertoli cells were assessed.

When the number and surface area of the SSCs colonies were analysed, according to the different GnRHa treatment doses, the highest effects were observed in group B, which received 1 µg/ml GnRHa. The highest treatment dose (4 µg/ml GnRHa, group D) negatively affected SSCs colonization (both number and surface area).

It seems that treatment with 1 and 2 µg/ml GnRHa had a proliferative effect on SSCs or at least reduced apoptotic cell death. However, 1 µg/ml GnRHa was more effective in these aspects. Treatment with 0.5 µg/ml GnRHa could not stimulate SCCs proliferation or prevent SSCs apoptotic cell death. Although this treatment dose did not have any inhibitory effect on SSCs colonization. Some researchers demonstrated that after homologous transplantation, colonization of donor-derived SSCs in the seminiferous tubules of the recipient was increased if the recipient received GnRH (Ogawa *et al*., 1998; Ogawa *et al*., 1999; Dobrinski *et al*., 2001). It is reported that, GnRH trearment before and after homologous SSCs transplantation decreases testosterone secretion from testes because of the pituitary gland GnRH-Rs down-regulation and low testosterone levels in the recipient testis tissue supports SSCs implantation and colonization (Ogawa *et al*., 1999). Although, GnRH direct effects on successful homologous SSCs implantation and proliferation in the recipient testis can not be completely excluded. In the present study, the highest GnRHa treatment dose decreased both the number and surface area of the SSCs colonies. Probably, high GnRHa dose caused GnRH-Rs down-regulation which are present on the germ cells, like what occurs in the pituitary gland. Adding 4 µg/ml GnRHa to the media culture may have stimulated SSCs apoptosis, like what occurs in rat atretic follicle and corpus luteum cells (Billing *et al*., 1994; Saragueta *et al*., 1997) or human atretic endometrial cells (Hsien-Ming *et al*., 2009). Finally, adding 4 µg/ml GnRHa to the media culture may have stimulated SSCs differentiation, because according to Anjamrooz *et al*. (2006) SSCs that enter the differentiation process can not create colonies. With regard to the results of the present study, probably lower GnRHa concentrations in the media culture can not carry out these effects.

Shafiei *et al*. (2013) reported that adding Colony stimulating factor-1 (CSF-1) to the SSCs co-cultural media with the sertoli cells increased SSCs colonization in comparison with the control group in a direct way (CSF-1 affects SSCs, no sertoli cells). In the present study, GnRHa affects SSCs in a direct way too. In two reports, adding FSH (Narenji Sani

et al., 2012a) and testosterone (Narenji Sani *et al*., 2012b) to the bovine SSCs co-cultural media with sertoli cells increased SSCs proliferation on an indirect manner (FSH and testosterone affects sertoli cells and the cells in turn produce and secrete growth factors that affects SSCs). In the similar researches on the mice SSCs, Koruji *et al*. (2009) demonstrated that adding GDNF to the SSCs co-cultural system with the sertoli cells increased SSCs colonization in comparison with the control group in a direct way. Anjamrooz *et al*. (2006) reported that adding EGF to the mice SSCs co-cultural system with the sertoli cells increased SSCs colonization in comparison with the control group on either direct and indirect ways. Pellegrini *et al*. (2003) demonstrated that adding Bone morphogenetic protein 4 (BMP4) to the mice SSCs co-cultural system with the sertoli cells decreased SSCs colonization in comparison with the control group. By adding activin (Mather *et al*., 1990) and inhibin (van dissel-Emiliani *et al*., 1989) to hamster SSCs co-cultural system with the sertoli cells, SSCs colonization increased and decreased in comparison with the control groups on the direct and indirect ways respectively.

The results of the present study showed a negative relation between the number and diameter of SSCs colonies. It seems that when SSCs colonies number is decreased, their diameter is increased, because some of the colonies join together and form a larger colony.

In conclusion, the results showed that 1 μg/ml GnRHa has the highest effect on SSCs proliferation in short- time co-culture with the sertoli cells.

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مطالعه تاثیر هورمون آزادکننده گنادوتروپین (GnRH) سلول‌های بنیادی اسپرماتوگونی گاو

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چکیده

شناخت عوامل محرک تکثیر سلول‌های بنیادی اسپرماتوگونی در محیط کشت، راه‌های ارزشمندی را برای مطالعه بیولوژی پایه این سلول‌ها فراهم می‌کند. هدف مطالعه اخیر ارزیابی تاثیرات یکی از آنالوگهای GnRH (alareline acetate) بر کلونی‌سازی سلول‌های بنیادی اسپرماتوگونی در کوتاه مدت در محیط هم‌کشتی با سلول‌های سرتولی بود. از پنج گوساله سه ماهه نژاد هلشتاین برای جداسازی سلول‌های اسپرماتوگونی و سرتولی استفاده شد. برای جمع‌آوری سلول‌های اسپرماتوگونی از روش‌های هضم آنزیمی استفاده شد. سلول‌های بنیادی اسپرماتوگونی به مدت پانزده روز با سلول‌های سرتولی کشت داده شدند و اثرات دزهای مختلف GnRHa (۰ /۵,۱,۲,۴µg/ml) بر کلونی‌سازی سلول‌های بنیادی اسپرماتوگونی ارزیابی شد. تاثیرات GnRHa بر تکثیر سلول‌های بنیادی اسپرماتوگونی وابسته به دز بود. در پایان مشخص شد که دز ۱ µg/ml GnRHa بهترین اثر را بر کلونی‌سازی سلول‌های بنیادی اسپرماتوگونی در مقایسه با گروه کنترل دارد. بالاترین دز درمانی (µg/ml ۴GnRHa) دارای اثرات منفی بر کلونی‌سازی سلول‌های بنیادی اسپرماتوگونی در مقایسه با گروه کنترل بود.

واژگان کلیدی: هورمون آزادکننده گنادوتروپین، گوساله، سلول‌های بنیادی اسپرماتوگونی، سلول سرتولی، محیط هم کشتی